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Award Number: DAMD17-01-1-0289

TITLE: Brain-Sparing Strategy for Breast Cancer Treatment and Prevention

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REPORT DATE: July 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 074-0188*



| 1. AGENCY USE ONLY (Leave blank) | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | July 2002 | Annual (1 Jul 01 - 30 Jun 02) |

**4. TITLE AND SUBTITLE**
Brain-Sparing Strategy for Breast Cancer Treatment and Prevention

**5. FUNDING NUMBERS**
DAMD17-01-1-0289

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**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**
Report contains color

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**12b. DISTRIBUTION CODE**

**13. ABSTRACT** *(Maximum 200 Words)*

Tamoxifen is a mainstay of hormonal therapy for breast cancer patients, but its antagonism of estrogen actions in the brain has the potential of depriving woemn of the beneficial effects of estrogen on affect and cognition. Tamoxifen methyl iodide (TMI) was designed to obviate this potential problem through limited access to the brain confirmed by the permanent charge on the mocleule. To prove that TMI and its metabolites do not penetrate the brain and / or interact with brain estrogen receptors, we have employed radioactive TMI and shown that brain levels were low to undetectable, as compared to high uptake and accumulation with tamoxifen. Furthermore, following 3 weeks of continuous exposure, there was no evidence for occupancy of brain ER in the TMI treated animals as compared to highly significant occupancy with tamoxifen and estradiol. These results , combined with the efficacy of the compound in animal models of human breast cancer, support further development and evaluation of TMI in human subjects.

**14. SUBJECT TERMS**
breast cancer, Brain-Sparing Strategy, Tamoxifen

**15. NUMBER OF PAGES**
12

**16. PRICE CODE**

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Unlimited |

## Table of Contents

## Introduction

The purpose of the studies described was to evaluate the interaction – or lack thereof – of a permanently charged derivative of tamoxifen (TAM) with brain tissue in general and with brain estrogen receptors (ER) in particular, using the rat as a model. This research stems from recent findings supporting the use of TAM as a preventive agent for healthy women at high risk for breast cancer, in addition to its established use as adjuvant therapy in women diagnosed with breast carcinomas. At the same time, accumulating research results from clinical and animal studies suggest that TAM interacts with ER in the brain and that this interaction may unravel the positive effects of estrogen on cognition and mood. We have argued that a tamoxifen analog designed not to penetrate the blood brain barrier (tamoxifen methyl iodide, TMI) may have the positive anti-cancer qualities of tamoxifen without the negative effects related to blockade of estrogen receptors. Using radioactively labeled TMI, TAM and estrogen analogs we have attempted to prove that this compound is indeed hindered in its penetration into the brain and does not significantly occupy brain ER even after several weeks of continuous treatment with doses relevant to inhibition of breast cancer growth. Both dissection and autoradiography were used to characterize the interaction of the compound with the brain.

## Body

1. The first task was to synthesize TMI for the acute as well as chronic studies. Dr. Gibbs from the chemistry group in our department has accomplished the synthesis as follows:

a. First (small scale) synthesis: Methyliodide (9.2 $\mu$l, 0.148 mmol) was added to a solution of Tamoxifen (50 mg, 0.134 mmol) in 1 ml of dichloromethane at 0 °C. After 3 hours at 0 °C a white precipitate had formed and no Tamoxifen remained by TLC analysis (10 % methanol / chloroform). Ice-cooled ethyl acetate was added (2 ml) and the white solid collected by filtration, rinsed with ice-cooled ethyl acetate (3 x 2 ml) and dried under vacuumn to give 57 mg of quaternarized Tamoxifen. Purity was checked by nmr yieldig the expected spectrum:
$^1$H-nmr ($CD_3OD$); 7.35-7.10 (10 H, m, 10 ArH), 6.83 (2H, d, 2 ArH), 6.66 (2H, d, 2 ArH), 4.34 (2H, undefined triplet, $CH_2$), 3.77 (2H, t, $CH_2$), 3.22 (9H, s, 3 x $CH_3$), 2.45 (2H, q, $CH_2$) and 0.91 (3H, t, $CH_3$).

b. Second (upscaled to gram amount) synthesis: 1.0 g of Tamoxifen (2.69 mmol) in 5 ml of dichloromethane, was cooled to 0 °C. Methyl iodide was added dropwise (180 $\mu$l, 2.96 mmol) over a period of 10 minutes. After complete addition reaction was maintained at 0 °C for 3 h, over which time a heavy white precipitate formed. The solid was removed by filtration, and washed thoroughly with ice-cold dichloromethane (3 x 5 ml), dried under vacuum and recrystalized from methanol to give 1.14 g (82.5% yield) of quaternized Tamoxifen. One gram from this synthesis was sent to Innovation Research for pellet preparation for the chronic studies. Tritium labeled TMI for the acute studies was purchased from ARC.

2. The second task was to measure the radioactivity in the brains of animals subjected to intravenous administration of a radiolabeled form of TMI and, for comparison, radiolabeled tamoxifen. The original plan was to have the radio-labeling of TMI performed at the National tritium labeling Facility located at LBNL. However, the facility was closed down shortly after the grant approval and this required us to turn to a commercial vendor for the labeling. Tritium labeled TMI (specific activity 80 Ci/mmol) was thus purchased from ARC. Tritiated tamoxifen (specific activity 84 Ci/mmol) was purchased from Amersham. The two compounds were injected (100$\mu$Ci/animal in the tail vein) into groups of rats which were decapitated at different time points post-injection. As expected, we found high concentrations of tamoxifen in the brain; with the brain:blood ratio exceeding 1 and increasing over time after injection, suggesting accumulation and retention of the agent in brain tissue (table 1). As can be seen from the table, brain levels of TMI were negligible at all times and could be easily accounted for by TMI in blood vessels in the brain (4%).

**Table 1. Brain penetration of TAM and TMI following intravenous injection of the tritium labeled drugs**

| Agent | Time | Brain Radioactivity | Blood radioactivity | Brain / blood Ratio |
|---|---|---|---|---|
| [$^{3}$H]TAM | 15 min | 1202+/-300 | 129+/-21 | 9.3 |
| [$^{3}$H]TMI | 15 min | 43+/-2 | 521+/-50 | 0.08 |
| [$^{3}$H]TAM | 60 min | 602+/-12 | 79+/-1 | 7.6 |
| [$^{3}$H]TMI | 60 min | 42+/-2 | 232+/-30 | 0.18 |

Animals were killed 15 or 60 minutes after injection. Brain radioactivity is represented by dpm/mg of cortex sampled from the frontal pole, blood radioactivity is also in dpm/mg.

Film autoradiography following a 2 months exposure showed widespread distribution of [$^{3}$H]Tamoxifen in the brain (figure 1). The autoradiograms from TMI injected animals were invisible (film background level) even after this long exposure. The distribution of Tam in the brain was not restricted to ER rich regions; in agreement with literature suggesting multiple sites of action for tamoxifen (e.g. Wiseman 1994) as well as recent results from Brookhaven National Laboratory using Carbon-11 labeled tamoxifen in humans. This group also showed that $^{11}$C-tamoxifen uptake in the brain was not blocked by estradiol pretreatment (2000). At this point in the funding period, tritium sensitive film was pulled from the market by the only manufacturer; so that autoradiographic work with tritium labeled drugs was not possible. With this in mind, rather than proceed with the reminder of the task 2 experiments involving estradiol pretreatment and tritiated TAM and TMI injection, we have decided to proceed to task three. Unlike TAM and TMI, which can only be labeled with tritium for autoradiography, estrogen analogs with radioactive tags other than tritium (e.g. Fluorine -18 or iodine -125) could be prepared by our radiochemists.

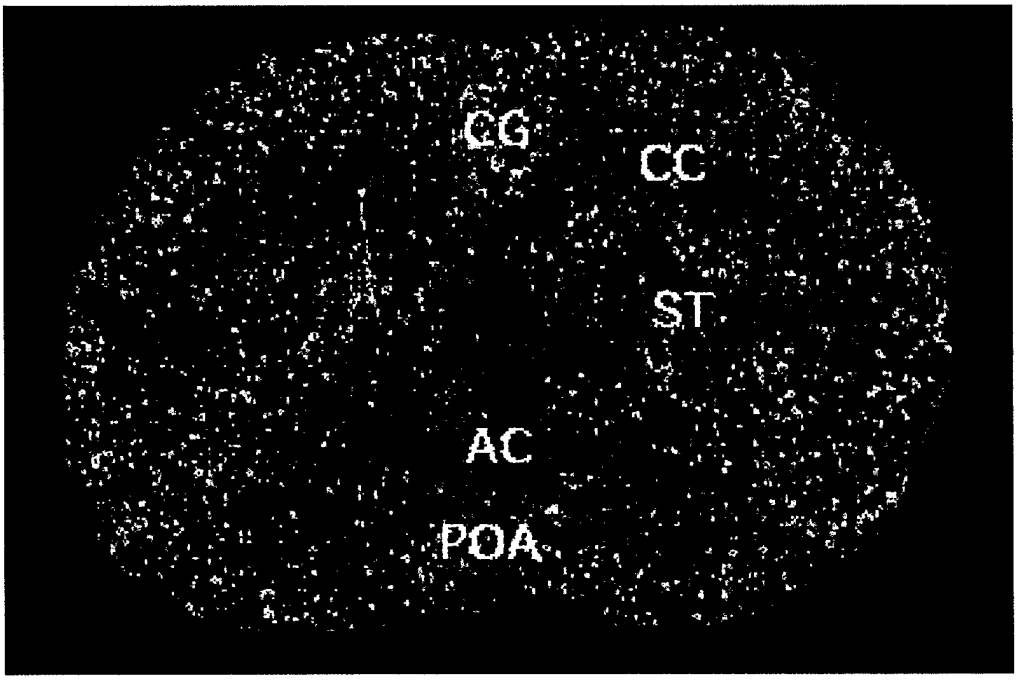


Figure 1. Regional distribution of tamoxifen in the rat brain

Animals were injected with 100$\mu$l tritiated tamoxifen and killed 60 minutes later. Twenty $\mu$ thick cryostat sections were apposed to tritium sensitive film (Amersham) for 60 days. After developing the film using Kodak developer and fixer, the film was scanned on a large bed Umax scanner. The rainbow scale (purple,blue =low, orange-red=high) was used to pseudocolor the auotradiogram. AC=anterior commisure, CC=corpus callosum. Note the relatively low accumulation in these white matter tracts. CG=cingulate cortex, POA=preoptic area, ST=striatum. Note the similarity in radioactivity levels in the ER rich POA , the cortex, with moderate concentrations of ER, and the ER- poor striatum.

3. The third task was to treat animals chronically (3 weeks) with TMI and determine whether under such conditions, the compound or a metabolite actually enters the brain and interacts with brain ER. For this purpose, slow release pellets with TMI were commissioned from Innovation Research of America using our locally synthesized TMI and ovariectomized female rats. We have chosen to purchase ovariectomized animals from Charles River rather than perform the surgery ourselves as originally proposed since we found these animals are now commercially available from this vendor at a modest prive increment over normal females; obviating the need for approval by the LBNL animal use committee Control animals received a molar-equivalent

amount of tamoxifen in similar pellets. Estrogen pellets were used for positive "total occupancy " control and blank pellets were used as "0 occupancy " controls.

Following this chronic exposure, animals were injected with a radiolabeled form of estradiol (F-18 or I-125 labeled) and brain, blood and uterus sampled, weighed and counted

In the first experiment we used F-18 labeled estradiol prepared by the DFI chemists. Animals were injected with 200$\mu$Ci in the tail vein and sacrificed 60 minutes later. The hypothalamus, preoptic area, cortex and hypothalamus were dissected, counted and weighed.

As expected, we found the highest levels of radioactivity in the brains of ovariectomized females with blank pellets. Since the cerebellum does not contain ER, the ratio of region/cerebellum was used to assess specific ER binding in the various regions and treatment groups (figure 2). TMI treated animals had brain radioactivity levels and region/cerebellum ratios indistinguishable from those seen in ovariectomized controls, supporting the notion that TMI or metabolites did not occupy estrogen receptors in the brain. Estradiol and tamoxifen pellets produced significant decreases in the level of radioactivity and region/cerebellum ratio; indicating significant occupancy of brain receptors.

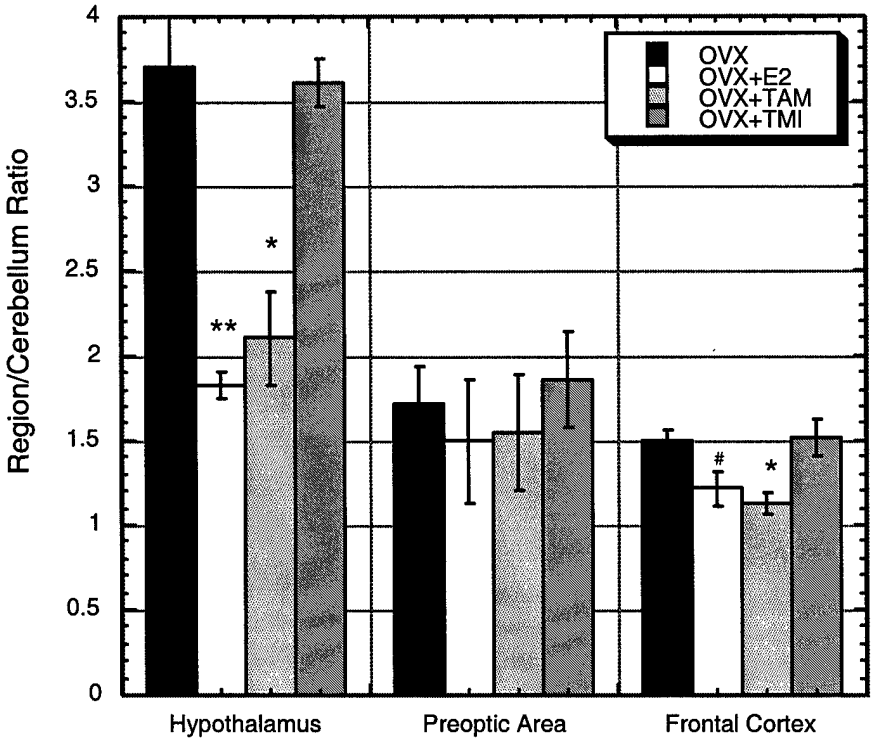


Figure 2. Regional Brain Estrogen Receptor occupancy by estradiol, tamoxifen and TMI.

Groups of animals were injected with Fluorine -18 labeled estradiol and decapitated 60 minutes later. OVX= control ovariectomized animals implanted with blank pellets. OVX+E2; ovariectomized females implanted with 21 day controlled release 17beta estradiol pellets. OVX+TAM, ovariectomized females implanted with 21 day controlled release tamoxifen pellets. OVX+TMI, ovariectomized females implanted with custom-made 21 day release TMI pellets in equimolar amount to the tamoxifen pellets.

*,**=significantly different compared to OVX at the $p<0.05$ or $p<0.01$ level, respectively. #=borderline significance ($p<0.1$).

The region with the highest ratio to cerebellum as well as the biggest effect of estradiol and tamoxifen was the hypothalamus, which contains the highest concentrations of ER in the brain. Unfortunately, the absolute levels of radioactivity combined with the short half life (110 min) of Fluorine -18 did not allow for autoradiographic detection on film. To facilitate the latter, we have

synthesized an Iodine-125 (half life 60 days) labeled analog of estradiol used by MacLusky et al. for brain ER auotradiography. This agent was used in the last experiment performed to date. Animals were injected with radioactive estradiol and brains frozen for sectioning and autoradiography, which are pending at the time of submission of this report and should be completed within the first 6 months of the 2nd year..

Due to the difficulty of performing tritium autoradiography and the conclusive nature of the acute data obtained so far, we propose to use the last 6 months of the grant period and funding to advance into humans, performing feasibility studies on labeling ER in the human brain with the F-18 labeled estrogen analog used in our rat studies (18-FES), in preparation for testing TMI in humans. The suggestion to initiate human studies was brought up in the initial review of the current grant. We have written and received approval for human protocols to use 18-FES and PET to label ER in the brains of postmenopausal women on TAM or hormone replacement therapy and would like to use the last six months of funding to advance this project through the feasibility stage (scan and interpret data from 4-5 subjects/group). Alternatively, we would continue our attempts to find a replacement for the tritium sensitive film and perform the remaining studies described in task 2.

**Accomplishments**

1. Synthesis of gram amounts of TMI with high (>80%) yield
2. Proof that TMI does not appreciably penetrate the blood brain barrier upon acute intravenous administration
3. Accumulating evidence that even after 3 weeks of exposure, TMI treatment does not result in appreciable occupancy of estrogen receptors in the brain.

The results accumulated so far were accepted for a presentation at the annual American Society for Neuroscience meeting in Orlando, Nov. 2-7 (appended)

**Conclusions**

A permanently charged derivative of tamoxifen does not accumulate in the brain after intravenous administration. Following three weeks of continuous exposure, brain estrogen receptors in key regions of the brain do not appear to occupied; in contrast with tamoxifen and estradiol, which block more than 50% of the uptake of radioactivity in key estrogen-receptor

containing brain regions. The quantitative autoradiographic analysis of the effects throughout the brain is to be completed in year 2 of the grant period.

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Appendix: SFN 2002 in press abtract no 11833

BRAIN PENETRATION AND ESTROGEN RECEPTOR OCCUPATION BY ESTRADIOL, TAMOXIFEN AND TAMOXIFEN METHYL IODIDE

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Antagonism of brain estrogen receptors (ER) by tamoxifen may increase vulnerability to depression and Alzhemimer's disease. Tamoxifen methyl iodide (TMI) is a quaternized tamoxifen analog designed to prevent brain penetration and ER occupation. To assess these parameters, [3H]tamoxifen and [3H]TMI were injected I.V. at 0.5mCi/Kg into groups of 4 ovariectomized (OVX) rats. Brain and blood samples were harvested 15 or 60 min later. Tamoxifen injected animals had 600-1200 dpm/mg in the brain as compared to 37-47 dpm/mg in the TMI injected animals. Brain/blood ratio was 7.6-9.3 for tamoxifen and 0.08-0.18 for TMI. In a 2nd study, OVX rats were implanted with pellets containing estradiol, tamoxifen, TMI or blank powder. Three weeks later, groups of 4 animals were injected with radioactive (F-18) fluoroestradiol (0.5 mCi/Kg) and killed 5, 20 or 60 minutes later. Brain samples were dissected and counted. As expected, OVX controls had a high (3.7+/-0.38) hypothalamus -to -cerebellum binding ratio at 60 min. Estradiol and tamoxifen resulted in a significant reduction of this ratio to 1.8+/-0.08 and 2.1+/-0.27, p=0.001 or 0.003 compared to OVX respectively, while TMI treated animals had a high (3.55+/-0.2) indistinguishable from OVX. Cortical ER were similarly occupied by estradiol and tamoxifen (p=0.01, 0.04 compared to OVX) but not TMI, demonstrating that TMI treatment does not result in brain estrogen receptor occupancy even after three weeks of continuous administration. These results support further development of TMI as a brain-sparing breast cancer treatment and prevention modality.

Supported by: DAMD17-01-1-0289.